THE LOCALIZATION OF RIBO-NUCLEIC ACID IN THE OVARY OF THE PREPUBERTAL RAT

by

WALTER SAMPSION VINCENT, JR.

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June 1948
APPROVED:

[Signature]

Associate Professor of Zoology
In Charge of Major

[Signature]

Chairman of Department of Zoology

[Signature]

Chairman of School Graduate Committee

[Signature]

Dean of Graduate School
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THE LOCALIZATION OF RIBO-NUCLEIC ACID
IN THE OVARY OF THE PREPUBERTAL RAT

INTRODUCTION

Studies too numerous to mention and many hypotheses based on them have been made regarding the origin of the germ cell. Recent studies (Slater and Dornfeld, 1945) have indicated that the ovum, in mammals at least, is differentiated from the cells of the germinal epithelium. This takes place in the form of invaginated cell masses, called germinal nests, which are proliferated from the epithelium and soon cut off into discrete groups by growth of the ovarian connective tissue. It is from one of the cells in these nests that the ovum is differentiated, with the remainder forming the follicle which surrounds it.

Egg cells in general contain large amounts of cytoplasm, and apparently the effect of some stimulatory mechanism on one particular cell sets into action the process of protein synthesis which accounts for the cytoplasmic growth which differentiates the ovum from its fellows. The recent works of Caspersson and co-workers (see Caspersson, 1947, for review) have demonstrated unequivocally that protein synthesis is invariably coupled with an increased amount of ribose nucleic acid in the cytoplasm, (cf. ibid. p. 140 regarding protein synthesis
in the egg).

Observations in this laboratory (Dornfeld and Whitlock, 1945) have suggested that a localization of germinal proliferation exists in the oviducal-suspensory ligament area of the ovaries of young albino rats. This observation has been confirmed and extended in this study.

Particularly, this study undertakes the localization of ribo-nucleic acid in the ovary of the prepubertal rat in an attempt to determine in some measure the agents dealing with the differentiation of the mammalian egg within the ovary.

The observations presented have been based on a study of some 50 ovaries from 33 rats aged 1 to 60 days post-partum. These ovaries have been treated with a variety of histochemical procedures which will be described.

A series of 70 ovaries prepared with the osmium reduction method of Levi (age group 0 to 60 days post-partum) and another series of 47 ovaries fixed with Goldsmith's mixture and stained with Iron Hemotoxylin (age 5 to 10 days) have been available for further comparison. Acknowledgment is given to Bertha D. Cutress and E. J. Dornfeld respectively for placing this material at the author's disposal.
Animals and animal care:

The animals used in this study were the offspring of seven male and ten female white rats of the Sprague-Dawley strain. The breeders were selected from mature animals of a group inbred in this laboratory for three generations. The animals were kept in wire cages, four adults to a cage. Two to three days prior to parturition the pregnant females were isolated in individual cages with excelsior as nesting material. Food and water were provided *ad libidum*. The stock diet used was compounded as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>yellow whole corn meal</td>
<td>38.0%</td>
</tr>
<tr>
<td>whole wheat flour</td>
<td>32.0%</td>
</tr>
<tr>
<td>powdered skim milk</td>
<td>20.0%</td>
</tr>
<tr>
<td>whole alfalfa meal</td>
<td>6.0%</td>
</tr>
<tr>
<td>cod liver oil</td>
<td>2.0%</td>
</tr>
<tr>
<td>yeast</td>
<td>1.0%</td>
</tr>
<tr>
<td>calcium carbonate</td>
<td>0.5%</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

This diet was supplemented with greens (lettuce trimmings) twice weekly; also, for one week prior to parturition and for one week after, the females were given a pellet (ca. 0.75 gm) daily of dried liver-yeast concentrate formulated as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>irradiated brewer's yeast</td>
<td>50.0%</td>
</tr>
<tr>
<td>dessicated liver powder</td>
<td>50.0%</td>
</tr>
<tr>
<td>cornstarch and water to form</td>
<td></td>
</tr>
<tr>
<td>tacky mass</td>
<td></td>
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</tbody>
</table>
The strain used for this study was difficult to handle, particularly since very young rats were to be used. Many lactating females ate their young within the first 48 hours after birth. Only eight litters of a total of 49 born during the five-month period of October 1947 to March 1948 survived beyond two days. The diet was not the source of the difficulty, since three other strains of rats used in the laboratory and maintained on the same food produced large, healthy litters, even without supplement.

The breeding and age determination for the animals in this study was that described by Slater (1945). The standardized gestation period of 22 days (528 hours post-coitum) was adopted as the reference point.

Microtechnical methods:

Animals up to 20 days post-partum were killed by injecting the fixative directly into the brain with a hypodermic needle. After 20 days, when the skull had become sufficiently ossified to make this method difficult, the animals were anesthetized with ether, the abdominal cavity opened and the ovaries removed prior to the death of the animal.
Zenker's fluid\textsuperscript{1} was used throughout the entire series of animals. Fixation in all instances was excellent. Both the acetic and formol mixtures were used in the first members of the series, but comparisons indicated that the formol mixture gave the greatest cytoplasmic detail so it alone was used later. Other fixatives were found to be unsuitable. Goldsmith's fluid, although an excellent fixative for embryonic tissue, did not give uniform staining results with methylene blue, particularly after ribonuclease treatment.

The ovaries were fixed for six to twelve hours, washed in tap water overnight, dehydrated in several changes of dioxane and imbedded in 56-58\degree paraffin. By cooling the paraffin block and knife with dry ice to prevent collapse, sectioning was done serially at 5 or 7\,\mu.

From the one ovary of each pair, alternate sections were placed on each of two slides, the first to be treated with ribonuclease and the second to be used as control. The other ovary of selected pairs (approximately one for each five-day age interval) was mounted by placing every

\begin{itemize}
\item Zenker's fluid has the following formula:
\begin{itemize}
\item potassium bichromate \quad 2.5 \,\text{gm.}
\item bichloride of mercury \quad 5.0 \,\text{gm.}
\item sodium sulphate \quad 1.0 \,\text{gm.}
\item water \quad 100.0 \,\text{cc.}
\end{itemize}
\end{itemize}

Add 5 cc. of formalin or glacial acetic acid just before using.
tenth section on each of five different slides in such a manner that the consecutive slides formed a sequence of five sections. These slides were then used as follows: (a) Feulgen reaction, (b) incubation without ribonuclease, (c) ribonuclease treatment, (d) staining in methylene blue at pH 4, (e) staining in methylene blue at pH 6.5. The remainder of the sections were used for control staining in various buffered solutions of methylene blue.

**Histochemical methods:**

The major conclusions in this study have been drawn from the application of the Brachet technique for basophilia removed by ribonuclease. This technique has been modified according to Dempsey and Wislocki (1946), utilizing the crystalline ribonuclease as prepared by Kunitz. Such ribonuclease was obtained from Armour Laboratories. Essentially the technique was as follows: The sections were mounted as above, deparaffinized in xylol and run to water through graded alcohols. One of the pair of slides was then placed in 50 cc. of 0.1 per cent ribonuclease (1 mg./cc.), the other placed in a similar amount of distilled water, and both incubated for three hours at 60° C. At the end of this period the slides were removed and each washed thoroughly in separate battery jars of tap water so as to remove completely the soluble products of
the enzyme digestion. Each slide was then rinsed in distilled water and both stained simultaneously in the same staining dish of Unna's alkaline methylene blue prepared as follows and diluted 1:4 with tap water.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>methylene blue chloride</td>
<td>1 gm.</td>
</tr>
<tr>
<td>potassium carbonate</td>
<td>1 gm.</td>
</tr>
<tr>
<td>distilled water</td>
<td>100 cc.</td>
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</table>

The slides were left for thirty minutes, then washed in tap water and dehydrated rapidly through successive alcohols to 95 per cent. They were then destained in 50 cc. of 95 per cent alcohol to which 1 cc. of a 10 per cent resin mixture in absolute alcohol had been added. Both slides were destained simultaneously in this mixture, washed in 95 per cent alcohol, absolute alcohol, cleared in xylol, and mounted permanently in balsam under #1 coverslips. Comparative studies of the stained areas of adjacent sections were then made as will be described later.

To obtain adequate controls, and to throw more light on the identity of the basophilic substances demonstrated above, and on their production, the following series of staining techniques were carried out on the slides mounted with every tenth section. (a) The Feulgen reaction: This test, specific for deoxyribose-nucleic acid, was carried out exactly according to the directions given by Cowdry (1948) except for a minor modification in the preparation
of the leuco-fuchsin. This was treated with Norite after the 24-hour bleaching period had elapsed, as suggested by Lillie (1946, p. 66). The same sample of basic fuchsin was used in all reactions.

(b and c) The Brachet ribonuclease test as described above.

(d and e) The controlled pH method as adapted from Dempsey and Singer (1946). This treatment was as follows: 50 cc. of acetate buffer were diluted to 1000 cc. with distilled water. To this was added 160 mgm. of methylene blue chloride. Two pH levels were used, 4.0 and 6.5, the pH of the mixture being checked before and after staining by a glass electrode pH meter. This dilution gave an ionic concentration of about 0.01 and a methylene blue concentration of about $5 \times 10^{-4}\text{M}$. Sections were run to water and stained for one half hour in 50 cc. of the above mixtures, dehydrated quickly in 95 per cent and absolute alcohol, cleared in xylol, and mounted permanently in balsam.

(f) Straight controls by staining directly in Unna's alkaline methylene blue mixture. This involved treatment directly in methylene blue as described in the ribonuclease method above except that the slides were not incubated. The results were identical with those obtained from the sections incubated in distilled water for the three-hour period.
Specificities of staining techniques used:

The absolute specificity of the ribonuclease technique has been questioned by many authors (see Danielli, 1947; Stowell and Zorzoli, 1947). Most agree, however, (except Danielli) that with adequate controls, the technique can be considered qualitatively fairly accurate. The chemistry of the enzymatic action is not known in detail. The crude extracts used by Brachet were obviously contaminated with other enzymes, but the crystalline product obtained by Kunitz has been adopted by workers since that time and has given reproducible results in most instances. The action of ribonuclease is apparently that of a depolymerase with no phosphate activity, as the end products are invariably nucleotides with no inorganic phosphorus present. A certain proteolytic effect has been ascribed to ribonuclease by some workers (Catcheside and Holmes 1947), although it is claimed that the cytochemical picture obtained by slightly contaminated samples is no different from those which all evidence of proteolytic activity was removed (Kaufmann, 1947). A widely different activity of ribonuclease upon the same tissue is possible through the use of various fixing agents, as well as the type of buffer in the ribonuclease medium. This problem is discussed thoroughly by Stowell and Zorzoli (1947); Greenstein et al have shown the effect of ions on nuclease
activity (1946). Dempsey and co-workers have used ribonuclease with a large number of tissues and organs and, with a number of supporting techniques, have ascribed a fairly reliable specificity to ribonucleoproteins. (For detailed discussion see Dempsey and Singer, 1946; Dempsey and Wislocki, 1946). These techniques have involved mainly an analysis of staining reactions at controlled pH levels. A complete discussion of this will be given below. With the use of these confirming techniques, it has been assumed in this report that the basophilia removed by ribonuclease treatment has been the result of the removal of ribonucleic acid from its particular protein complex.

The Feulgen reaction, allegedly specific for desoxyribose nucleotides, apparently has the following action: Decolorized basic fuchsin (Schiff's reagent) is recolorized by the aldehyde remaining on the desoxyribose molecule following the hydrolytic removal of the purine or pyrimidine bases with warm N HCl. The color obtained in the tissue varies somewhat from violet to reddish purple, indicating that the tissue reaction is somewhat at variance with the normal in vitro reaction. Conn (1946) and Danielli (1947) give a thorough discussion of the chemistry postulated for the reaction. Stacey (1947) and also the Stedmans (review, 1947) have postulated a rather unspecific action which is not generally agreed to by other workers.
The controlled pH staining method is described fully by Dempsey and Singer (1946). Briefly it consists of staining various tissues at known pH from 1 to 9. The staining reaction is then measured colorimetrically, and graphs are prepared of the staining intensity to the pH level. Those organs known by quantitative methods (ultraviolet absorption and direct microanalysis) to contain large and nearly pure amounts of ribonucleoproteins, such as the Nissl bodies of nerve cells, were found to be totally chromatophobic to methylene blue below pH 4, the intensities at higher pH varying according to the concentration of the acid. Those areas which stained below a pH of 4 were shown to be the sulfuric-acid-ester types of compounds like the mucins, the mucopolysaccharides, and the matrix of cartilage; also the granules of mast cells, the particular agent responsible for the basophilia in this case being controversial. From the data gathered in the tests described above, Dempsey and Singer postulate that basophilia suppressed at a pH level below 4 must be that of the phosphoric acid ester. This, in fixed and well washed tissues could be only the nucleoprotein complex.

In this study no counterstain has been used with any of the techniques described, as adequate cellular detail was obtained with methylene blue alone. Such counterstains
as eosin were found to obscure some of the preciseness obtainable without it.

THE DEVELOPMENT OF THE OVARY AND CAPSULE

In order to obtain a clear picture of the orientation of the ovary and its allied structures, the following description of the development of the ovary up to the time of birth is presented. (Modified from Torrey, 1945, and Kellogg, 1941).

The region of the presumptive gonad is first defined on the dorso-lateral aspect of the coelom at 11 days post-coitum. At 13-1 1/4 days p.c. it is so oriented that most cellular proliferation takes place inward from the coelomic surface. At 15 days the ovary can be distinguished from the testis in a negative way in that the latter at this time will show well defined cords, while the former shows no definite structure. At 16 days the ovary consists of a dense central mass of cells bounded on the free surface by mesothelium (germinal epithelium). These central cells are commencing to show cord formation. Few mesenchymous cells are present. By the late sixteenth day the ovary begins to show characteristic chromophobie germ cells, but most are of the indifferent type. Very little vascular tissue is found. At 17 days a connective tissue web is well established throughout the ovary continuous
with the connective tissue supporting ligaments. The major change up to birth (22 days) is that of enlargement of this connective tissue web. A change of gross shape of the ovary from an elongated organ parallel with the body axis to rounded structure also occurs as will be described below.

During this latter period (17-22 days) the growth of the peri-ovarian capsule or sac has taken place. This consists of a delicate two-layered covering of the ovary by a portion of the mesenteries and ligaments associated with it. Most of the capsule is formed from the mesosalpinx, which is a mesentery associated with the degenerating mesonephros and the tubal ridge. At 17 days the ovary is situated as described above, with the longitudinal axis parallel with the body axis. The oviduct lies at the free edge of the mesosalpinx with the opening or ostium craniad and attached by the ligamentum ovarium to the diaphragmatic ligament and the anterior portion of the mesosalpinx. Caudad the oviduct and the mesosalpinx are attached to the ligamentum proprium. (The diaphragmatic ligament, continuous with the ligamentum proprium, is the suspensory ligament of this report). As mentioned above, between the seventeenth and the twenty-first days the ovary becomes reoriented in the following manner: The antero-lateral half moves ventrally and somewhat caudally, while the
postero-median portion of the elongated ovary moves laterally toward it, producing a U-shape. The mesovarium is by this movement transformed into the connective tissue mass in the fissure between the lobes. During this movement the mesosalpinx has been growing ventrally carrying the oviduct along its border. As a consequence of the oviduct being attached at or near the ends of the ovary, the oviduct is brought around its ventral aspect in a loop carrying the mesosalpinx with it. This results in the ostium being drawn into the space between the capsule-to-be and the ovary, coming to lie near the hilus in conjunction with the diaphragmatic portion of the suspensory ligament. With further growth and coiling of the oviduct the mesosalpinx is drawn over the ventral surface of the ovary until only a very small opening remains which persists in the adult.

Thus the ovary can be pictured as a somewhat kidney-shaped organ with the suspensory ligament as the point of attachment in the hilus and the free border lying ventro-laterally. The ostium is associated with the lateral side of the anterior portion of the ligament (diaphragmatic ligament) while the blood supply is associated with the dorso-caudal portion (corresponding to the ligamentum proprium). The oviduct, due to its extreme growth and attachment to the suspensory ligament at the ostium, becomes highly coiled in the antero-ventral aspect of the
OBSERVATIONS

Germinal epithelium:

The germinal epithelium of the ovary, at first cuboidal (-4 to 5 days post-partum), exhibits a progressive flattening in the prepubertal rat, becoming almost a pavement epithelium in the adult. This general picture is modified in localized areas, however, and it is apparently in the region of these modifications that the essential activity of the epithelium takes place. As early as -4 days p.p. a slight change toward a columnar type of epithelium is noted in the oviducal-suspensory ligament region of the ovary. This trend becomes increasingly evident in the older ovary. In ovaries up to five days p.p. other localized areas of columnar epithelium also occur.

The basophilia of the germinal epithelium roughly parallels the height of the cells—the columnar stages exhibiting greater cytoplasmic and nuclear staining intensity. The cytoplasmic basophilia is removed by ribonuclease. The nuclei of these cells exhibit strong basophilia with methylene blue and hematoxylin and also gives an intense positive Fuelgen reaction. The low cuboidal to flattened epithelium exhibits only faint to moderate cytoplasmic basophilia removed by ribonuclease and the nuclei
are moderately Fuellgen positive.

As mentioned above, in some of the younger ovaries isolated areas of the epithelium exhibit the columnar type of cell (Figures 5, 6 and 7). The regions are invariably co-existant with an area of intense basophilia of a diffuse nature removed by ribonuclease. This would suggest some local source of a large supply of the ribose nucleic acid complex. Reference will be made to this point below.

The regions of the oviducal-suspensory ligament and those mentioned above seem to be the points from which the Pflueger's cords of the young ovary arise (Figure 2). In the older ovary no such cords seem to be formed, but rather a local proliferation of small germinal nests takes place which are pushed peripherally from the point of origin (Figure 5).

Summary: The germinal epithelium in early stages shows little differentiation. After about five days p.p. the cells begin to transform from the cuboidal to the low cuboidal and flattened types, commencing with the free surface of the ovary and proceeding toward the attached region. This epithelium is only slightly basophilic and shows typical "resting" nuclei. On the other hand, the region of attachment, and particularly the area adjacent to the oviduct, is characterized by tall cuboidal to columnar epithelium, with intense cytoplasmic and nuclear basophilia.
Connective tissue basophilia:

As mentioned in the discussion on prenatal development of the ovary, the connective tissue undergoes considerable extension during the three days prior to parturition. This development continues after birth with the establishment of a fairly well defined layer beneath the germinal epithelium. The formation of this layer apparently cuts off the cords which have been produced by proliferation inward from the germinal epithelium. These are subdivided by the growth of the connective tissue into the germinal nests (Figure 2). With further development, the ovary becomes packed with follicles forming from these nests, until they completely fill it except in the region of the ostium (Figure 1). This results in tunica becoming closely applied to the flattened germinal epithelium in all regions except that mentioned. This is essentially the picture at puberty.

With the folding of the ovary and the establishment of the ovarian ligament as the connective tissue mass at the hilus, the connective tissue web becomes extremely active in the cortical region. Whether or not it is formed from the mass of the ligament or originates from separate mesenchyme has not been determined. Observations on the material studied indicates that the vascularization of the ovary takes place during this period from the region
of the ligamentum proprium. The follicles are surrounded by the connective tissue of the medulla from about seven days onward, and between the tenth and fifteenth day the interstitial tissue can be histologically differentiated. With increasing age the medullary connective tissue becomes widely distributed throughout the ovary with considerable portions being replaced by interstitial cells (Figure 11).

The connective tissue of the medulla may be considered continuous with the suspensory ligament. Structurally the ligament presents the same morphological picture throughout the entire period studied.

The above mentioned morphological changes are reflected in the physiological changes of the cells of the connective tissue as demonstrated by the techniques utilized. During the early growth periods of the ovary, i.e., the establishment of the connective tissue and the tunica propria, the cytoplasm shows intense ribonuclease removable basophilia coupled with intense basophilia of the nucleus and nuclear membrane. This nuclear staining is shown to be due to deoxyribose nucleic acid by the Feulgen test. In the region of the germinal nests of the young ovary the connective tissue of the medullary region is also quite active as indicated by the same reactions. With increasing age (10 days plus) this tissue loses its
activity as cytochemically demonstrated above and is apparently replaced in part by interstitial tissue.

As the connective tissue of the tunica becomes applied to the flattening germinal epithelium it exhibits a decreased basophilia in both the nucleus and the cytoplasm, indicating a lack of mitotic activity. This picture is not true in the region of the active columnar germinal epithelium. In this area the tunica is less well defined (Figures 5, 8 and 9), and the cytoplasm and nuclei are quite basophilic. Here numerous young oocytes and follicles can be found. In the areas adjoining this, young follicles, showing one or two layered epithelium and an attached thecal layer are numerous. The connective tissue of this region is considerably less active, as demonstrated by the above methods. This would seem to indicate that the young follicles, after obtaining the theca from connective tissue cells near the region of proliferation of the oocytes, are displaced peripherally by the growth of new ones (Figure 5). Such a picture is very pronounced in animals past 30 days p.p.

The medullary connective tissue mass is generally quite chromophobic to basic dyes.

Summary: In general, a decrease in basophilia of both the cytoplasm and nuclei of the connective tissue cells corresponds to a decrease in mitotic activity.
Actively proliferating connective tissue is associated with the germinal nests and young follicles throughout the entire ovary of the very young rat. In older ovaries the active tissue is found only in conjunction with the active regions of the germinal epithelium and is apparently utilized in formation of follicles. In the medullary region of the ovary the actively dividing connective tissue is replaced by (or transformed into?) interstitial tissue. The medullary connective tissue mass is only slightly basophilic.

**Interstitial tissue:**

Closely allied in distribution to the connective tissue is the interstitial tissue. These masses of cells are first apparent by the cytological methods used at 10 to 15 days. The distribution has been traced adequately in the section on connective tissue. As has been mentioned before, the interstitial cells first appear in regions of highly active connective tissue.

The cytoplasm of these cells is weakly basophilic, and with the fixatives used, somewhat granular to clear in appearance (Figure 11). All cytoplasmic basophilia is removed by ribonuclease. The nuclei show a moderate basophilia with methylene blue and one to five intensely staining small nucleoli. The nuclear membrane is also
quite definitely outlined. With the Feulgen test the nucleoli and nuclear membrane are moderately colored while the nuclear matrix is only faintly so. Little or no physiological change, as evidenced by the response to the cytochemical agents used, can be detected through the entire period studied.

**Oocyte basophilia:**

The development of the oocytes and their associated follicles has been described adequately and quantitatively by Slater and Dornfeld (1945). Briefly, the unilaminar follicle is first developed at about one day p.p., bilaminar ones at two days, multilaminar ones by four days and a few with antra by the eighth day. In animals past ten days follicles of all sizes can be found in any one ovary, from indifferent cells to large antrum containing one. Through the last period mentioned many follicles in various states of atresia may be found. This is particularly true in the indifferent cells at 0 and one day p.p. and in the larger follicles of the 20 to 30 days p.p. period. The matter of atresia of the younger cells will be discussed at some length below.

Particular attention has been devoted to the cytochemistry of the indifferent and unilaminar stages. The results are essentially the same whether these stages are
studied in the two day p.p. ovary or in one of 60 days p.p. The presumptive oocyte is first distinguished from the mass of cells proliferated from the germinal epithelium by an increase in the amount of cytoplasm (Figures 2, 8, 9 and 10). This increase, which results in an apparent loss of basophilia when compared to the densely-packed, deeply staining nuclei of cells with little cytoplasm, is apparently the source of the oft-mentioned statement that the germ cell can be distinguished by its lack of staining ability. Corresponding to this increase in cytoplasm is the appearance of one or two, usually the latter, large and moderately staining nucleoli in the nucleus. This is contrasted with the many, small, deeply staining nucleoli of the surrounding cells (Figure 10). The beginnings of a layer of follicle cells can be distinguished shortly after this modification. The cytoplasm of the oocyte is basophilic (ribonuclease removable), while the nucleus is more deeply stained. With the definite establishment of a layer of follicle cells, the oocytes possess a non-staining area around the nucleus. This is characteristic of the early unilaminar stage only, and can be found in ovaries of any age. Such observations have been made on Zenker-acetic, Zenker-formol, Goldsmith, and Levi fixed tissue when stained with methylene blue or iron hematoxylin. The lack of staining is not so pronounced with eosin. The
Feulgen test indicates a moderate concentration of the desoxy acid at the nuclear membrane, and the nuclear sap is also slightly positive. In both this and the previous stage the nucleoli are typically Feulgen negative, and are also negative to methylene blue following ribonuclease treatment.

With increasing growth of the follicle and the cytoplasm of the ovum, the nuclear sap and membrane become progressively less basophilic and Feulgen positive. Inversely proportional to this, however, is an increasing basophilia of the nucleoli, and a corresponding resistance to removal of basophilia by the ribonuclease treatment. Occasional nucleoli in follicles of late multilaminar and antrum stages will show a loss of basophilia following the treatment mentioned. This is demonstrated by a ring-shaped appearance of the nucleoli, the ring staining deeply with methylene blue and the center portion faintly or negatively basophilic (Figure 15). However, this is true of only a small minority of the nucleoli on any one section, the remainder being intensely stained (Figure 14). In spite of this intense basophilia exhibited even after ribonuclease treatment, these nucleoli are only faintly colored by the leuco-fuchsin following four minutes hydrolysis at 60° C. in N HCl, and only slightly more so after ten minutes in the HCl solution (Figure 13).
With this increase in size and basophilia of the nucleoli, a basophilic region becomes evident in the cytoplasm immediately adjacent to the nuclear membrane (Figure 12), whereas in very young follicles this region is chromophobic. Ova of atretic follicles do not show this basophilic region.

Summary: The oocyte is first recognized by the increase in cytoplasmic volume and the appearance of large nucleoli. These nucleoli are characteristically either negative or only faintly positive to methylene blue following ribonuclease treatment and are generally Feulgen negative. A nonstaining area immediately adjacent to the nuclear membrane distinguishes the oocyte in the unilaminar follicle. With the development of the multilaminar follicle, this chromophobic area is replaced by an area similar in extent but contrastingly basophilic. This is correlated with the development of large, deeply staining nucleoli which are generally resistant to ribonuclease treatment and only faintly Feulgen positive. Ova of atretic follicles are characterized by the lack of the above mentioned basophilic region surrounding the nucleus.

Basophilia associated with follicle development:

The follicle presents a physiological picture, as evidenced by the methods used, of rapidly multiplying
tissue. The first layer of follicle cells has been generally described as arising from indifferent cells of the group which produce the ovum. In the material studied this is characteristic of the young stages.

Multilaminar follicles present a uniform appearance of two actively proliferating layers of cells, the outer one of tall cuboidal cells adjacent to the basement membrane, and the other layer immediately surrounding the ovum (Figures 14 and 18). The cytoplasm of these cells is generally quite basophilic, with this basophilia being ribonuclease removable, and the nuclei are quite strongly Feulgen positive (Figure 13). Mitotic figures are numerous, with the polarization of the spindles suggesting these layers as the source of the rest of the follicular epithelium. With antrum formation this inner layer continues its activity and is presumed to be the source of cells of the egg hill.

Thecal tissue is first detectable at about five days p.p. It appears to be derived from the connective tissue and cannot be distinguished from it except for its tangential orientation to the surface of the follicle (Figure 14).

**Follicular atresia:**

This problem has been described and discussed by many authors, notably Kingsbury (1939). It will suffice
to say in the material studied follicular degeneration is first noticeable in the form of abnormal mitotic figures which give an intense Feulgen reaction. These figures are first seen along the borders of the antrum. As atresia progresses the cells closer to the basement membrane begin to evidence this phenomenon. The cells of the corona are among the last to be affected, particularly the inner layer. Both this and the layer adjacent to the basement membrane retain their active, cuboidal shape and radial orientation until late stages of atresia (Figure 18). Finally all orientation is lost and they are indistinguishable from the other degenerating follicular cells (Figure 20). The thecal layer enlarges by an increase of cytoplasmic volume and the nucleus becoming more diffuse. With this enlargement there is a slight decrease in both nuclear and cytoplasmic basophilia (Figure 20). Very little evidence of mitotic proliferation has been found in the theca at this stage.

Summary: The follicle is developed from the in-different cells proliferated from the germinal epithelium. The cytochemical reactions of the first layer formed as above indicates that it is the source of the follicular epithelium by mitotic proliferation. The theca is apparently modified from fibroblasts, and is normally distinguishable from the connective tissue stroma only by its
orientation around the follicle. Atresia in the follicle is first observed in the cells adjacent to the antrum, and progresses peripherally to the basement membrane. The active layers of cells retain their activity and orientation until the latest stages of degeneration. With the onset of atresia the thecal layer enlarges by hypertrophy, and apparently not by proliferation.

**Oviducal basophilia:**

Little mention has been made regarding the interrelations between the oviduct and the ovary. The earlier discussion on the development of the capsule has suggested the physical and spacial relationships of these two organs, and the following observations indicate as possible physiological relationship. As has been stated, the oviduct is associated with the diaphragmatic portion of the suspensory ligament. During the period of the covering of the ovary with the capsule, and the first 15 days p.p., the oviduct undergoes a remarkable elongation. No attempt has been made to determine this elongation quantitatively, but it is estimated to be in the order of 25 to 50-fold. This tremendous growth is reflected in the histochemistry of the epithelium and the surrounding layer of muscle cells. The epithelium is intensely basophilic, and the layer of muscular cells also. The supporting connective tissue possesses
practically no basophily.

The ostium which is enclosed in the capsule at about the nineteenth day p.c., also grows considerably during this period. During the early stages (until 15 days p.p.) it is intensely basophilic (Figures 2 and 4). With further development both the ostium and the oviduct become somewhat less intensely staining (Figure 5). All cytoplasmic basophilia in both the oviduct and ostium is removed by ribonuclease (compare Figures 2 and 3).

During the early stages of the development of the oviduct and the capsule, the duct lies in close proximity to the germinal epithelium of the ovary. It is in these regions that areas of active germinal epithelium and localized areas of ovarian basophilia are located. This can be traced along the surface of the ovary during the early post-partum period when the capsule is being formed. The area of close contact of the ovary with the oviduct exhibits the phenomenon mentioned. The area of the ovary in which the ostium enters the capsule in conjunction with the ovarian ligament particularly demonstrates this observation. (See Figure 2) In older ovaries, when the oviduct is no longer found in the capsule proper, it is in the region just mentioned that localized areas of proliferation will be found. This activity is particularly apparent in the region of the opening of the ostium as it
lies adjacent to the area of attachment of the ovary to the suspensory ligament in the hilar region (See Figures 1 and 5). All cytoplasmic basophilia in these locations is removed by ribonuclease.

Summary: The oviduct, which is in a period of rapid growth during the early post-partum period, is highly basophilic. This basophilia is confined to the regions of the oviducal epithelium and muscular layers and is removed by ribonuclease. During this same period, the ostium, located within the ovarian capsule, undergoes a similar phase of growth and basophilia. The areas of oviducal and ostial contact with the ovary are the locations of the active regions of the germinal epithelium discussed previously.

Metachromasia:

Two structures in the rat ovary manifest metachromasia. One, the mast cells, displays intense reddish granules in Unna's mixture in both control and ribonuclease-treated sections. Some of the inter-granular cytoplasmic basophilia is removed by ribonuclease. These granules give an intense blue but no metachromasia when stained in the dilute methylene blue at pH 6.5 and 4 (Figures 10 and 22). The other metachromatic bodies are found in the follicular epithelium of the ovaries of one animal. These bodies, which give a reddish-orange reaction, are generally within
a cyst-like structure formed by closely packed epithelial cells (Figure 16). The metachromasia is not altered by ribonuclease treatment. (Compare Figures 16 and 17) In-so-far as could be determined, these bodies resemble the bodies of Call and Exner found in the follicles of other animals. No mention is made in the literature of their occurrence in rats or of their exhibiting metachromasia.

**Pycnosis in young oocytes:**

During the period 0 to 5 days, a considerable amount of degeneration of young oocytes occurs. Quantitative studies in this laboratory (unpublished data) have indicated that this atresia affects as high as eight per cent of the oocytes in the ovary at this age, and remains at this level for the first two to three days post-partum. The mode of response of these pycnotic cells to the various histochemical agents used is presented with some observations as to the metabolic and physiological significance of the staining reactions involved.

The pycnotic cells, as the name would imply, are intensely basophilic, with both the cytoplasm and the nuclei taking up readily any basic dye (Figure 21). The cytoplasm is quite dense, and shows no structure with the fixatives used. It is usually quite shrunken from the surrounding cells. It is suspected that this shrinkage is
due to fixation although it is present in tissues fixed in a variety of fluids. Careful destaining of methylene blue or iron hematoxylin sections to the point of total loss of cytoplasmic staining reveals the nuclei with a pronounced membrane and many small, rod-like, densely staining bodies resembling pachytene chromosomes. Occasionally small densely stained bodies are found adhering to the nuclear membrane. With the Feulgen reaction the results are similar to that destaining method. The color intensity of the chromosome-like bodies approaches that of the metaphase chromosome (Figure 23). In later stages of degeneration the nuclei become spherical bodies recolorizing the leuco-fuchsin reagent intensely. Ribonuclease digestion of the sections also presents a picture similar to the above, in that the cytoplasmic basophilia is lost, as well as a considerable portion of the basophilia of the nuclear sap. The rod-like intensely stained bodies are quite visible without excess destaining (Figure 22).

The intensity of the nuclear membrane reaction in these pycnotic cells is quite similar to that of rapidly dividing cells, while normal oocytes of the same ovary demonstrated only faintly Feulgen-positive nuclear membranes. Further, no nucleoli have been observed in the pycnotic nuclei, another similarity to the dividing cell.

With the osmic acid fixative of Levi there is no
indication of any lipoid material in these cells. Therefore it must be concluded that the degeneration taking place is not of the "fatty degeneration" type. Further implications of these observations will be discussed later.

DISCUSSION

The process of oocyte differentiation may be divided into two steps: (a) the proliferation of groups of indiferent cells from the germinal epithelium, and (b) the actual differentiation of one of these cells into the definitive oocyte. These will be discussed perspectively below.

The process of proliferation has been noted to be localized, for the most part, in certain discrete areas of the epithelium. These have been described as the areas which are in close contact with the diaphragmatic portion of the suspensory ligament, therefore the zone of activity is unusually closely associated with it also.

A number of substances have been shown to stimulate mitosis, and these same substances have also been shown, in areas of "competent" tissue, to direct this stimulation into differentiated tissue. Outstanding in this field is the ability of a number of assorted agents to evocate neural tube formation in amphibian embryos. Among these
are fatty acids and sterol derivatives, including the sex hormones and carcinogenic compounds (See Needham, 1941 for review). Also very effective are nucleic acids and nucleotides. The recent experiments of Brachet (1947 a, and b) have demonstrated quite strikingly the effects of tissue fragments containing known amounts of ribonucleic acid. He found that a direct correlation existed between the number of successful evocations and the percentage of ribonucleic acid in the implant. These fragments were granules from liver and yeast obtained by centrifugation ("microsomes" of Claude), and alcohol-killed tobacco mosaic virus. The same agents, upon treatment with ribonuclease and subsequent washing, gave no evocations.

Several examples of unspecific stimulation of the germinal epithelium have been demonstrated. Stein and Allen (1942) noted an increase of mitotic activity upon injection of estrogens into the ovarian capsule. Bullough (1946) has confirmed and extended these observations on such widely unrelated forms as mice and minnows. Stein and co-workers (1947) have noted a depression of mitotic activity of the germinal epithelium following thyroxin injections into the ovarian capsule. Green and Zuckerman (1947) have observed that estrogens injected into monkeys over long periods (one year) apparently are effective in the stimulation of the early "growth and differentiation of
the ovum from the epithelium" and that androgens are effective in stimulating the early stages of ovum and follicle growth. The long period involved in this particular study, however, allows for a number of interacting factors which cannot be determined. Other agents have been found in this laboratory which produce a massive stimulation of mitotic activity in the germinal epithelium.

It can be seen from the above example that the stimulation of activity of the germinal epithelium is a relatively unspecific matter. As was mentioned, the normal active or stimulated areas in the rat ovary are highly localized. These areas correspond to the points where the germinal epithelium is in direct contact with the ostium and in the younger ovaries, to portions of the oviduct. Both of these regions are rapidly growing and indicate an abundance of ribonuclease-removable basophilia, presumably ribonucleic acid. In the adult ovary, the active areas are usually located in folds of epithelium between the corpora lutea. Such development is not surprising in the light of the above conclusions, as the luteal tissue is very rapidly dividing and shows an intense basophilia removed by ribonuclease.

The role of other agents, such as the sex hormones, pituitary hormones, and the thyroid obviously complicate the picture presented above. However, the description
given fits very nicely the conditions found in young animals which have in general been shown to be fairly refractory to hormonal influence.

The next problem, that of the differentiation of these epithelial proliferations into oocytes, is more complex. First of all, an investigation into the physiological changes which have been demonstrated proves profitable. The egg is different from the surrounding cells in possessing a hypertrophied cytoplasm and enlarged nucleoli. With progressing development the cytoplasm and nuclei became larger. The nucleoli also enlarge. The cytochemical reactions here are significant. First of all, the nucleus becomes progressively less basophilic and nearly negative to the Feulgen reaction. Such reaction suggests lack of synthesis of desosyribose nucleic acid within the cell. At the same time the nucleoli become progressively more basophilic. This is coupled with the appearance of a perinuclear basophilic area in the cytoplasm. Such a reaction is typical of rapid protein synthesis, such as occurs in nerve cells (Hyden, 1947; Bodian, 1947) and pancreatic cells (Noback and Montagna, 1947). Caspersson, on the basis of observations on the nucleoli of rapidly synthesizing and growing tissues, has postulated an interesting chain of events which takes place during protein synthesis involving the nucleolus as the major point of activity.
(review, 1947). The ovum undergoes intense protein synthesis. It differs from rapidly multiplying cells in that it does not undergo division. The apparent lack of the desoxy acid in the nucleus could account for this, as this acid is required for the normal duplication and pairing of chromosomes. This suggests that the initial step of differentiation involves the inhibition of the desoxy acid synthesizing ability. Experimentally, inhibition of desoxyribose nucleic acid has been accomplished in living tissue. Bodenstein and co-workers (1946, 1947, 1948), studying the effects of nitrogen mustards on amphibian development, found that an effective inhibition of mitosis occurred and was coupled with hypertrophy of the cells. These cells histologically resemble the oocyte, possessing a large diffuse nuclei and large intensely staining nucleoli. Quantitative determinations of the nucleic acid content revealed that synthesis of the desoxy acid had been effectively inhibited and that the synthesis of the ribose type was essentially unaltered (Bodenstein and Kondritzer, 1948). Also noted was the fragmentation of large nuclei and nucleoli in the hypertrophied cells. Such a phenomenon is noted in the later stages of follicular atresia in the rat, when the nucleus of the ovum presents such a picture. Prior to this stage the onset of follicular degeneration is marked by the disappearance of the
perinuclear band. This indicates that with the beginning of atresia there is a lack of necessary metabolites for the ovum to carry out its rapid synthesis. Later stages of starvation result in the nuclear and nucleolar fragmentation noted above.

It is probable, therefore, that the process of oocyte differentiation involves a reversible inhibition of the desoxy acid synthesizing ability within the cell. With this inhibition the supply of ribose nucleotides is devoted entirely to protein synthesis. Such a process would eliminate the possibility of yolk synthesis by cells surrounding the ovum, such yolk being then transported to it for storage as has been suggested by some authors (See Corner, 1932, p. 1577).

The supposition that the differentiation of the oocyte is caused by an inhibition of the desoxyribose nucleic acid synthesizing ability of a particular cell leaves two glaring questions to be answered. One, what is the nature of the inhibition, and the other, more fundamental, why is only one of a number of apparently identical cells susceptible to this inhibition? It is probable that the answer to either one of these questions will answer the other, but no explanation can be drawn from the results of the data at hand.

Two other observations are still to be dealt with:
(1) the resistance to removal of basophilia by ribonuclease in the nucleoli of older oocytes, and (2) the cytochemical reactions of pycnotic objects.

The first observation is not confirmed in the literature. Many authors have noted a decrease in basophilia of the nucleoli after ribonuclease treatment, and the formation of a ring-shaped structure with basic dyes and the Feulgen reaction. Caspersson has noted that, although the above staining reactions may be observed, the entire nucleoli absorb ultraviolet light uniformly at 2700 A. U. From such observations it has been postulated that the nucleoli are essentially a ribose nucleic acid complex surrounded by a thin shell of a desoxyribose nucleoprotein. Such a conclusion fits the observations made here, as in certain instances the nucleoli do present such a staining reaction as has been described by others. As this action of ribonuclease is, however, not uniform, it seems probable that the highly basophilic outer layer of the nucleolus is an effective barrier against enzymatic action on the substrate within; or, if such enzymatic activity is possible even without direct access to the substrate, (as has been suggested by Rothen, 1947) the products of the degradation may not be permeable through the outer layer. The occasional observation of a nucleolus giving the ring-shaped reaction after ribonuclease treatment must be due then to
the bisecton of the organelle during the microtoming of the tissue, making the contents available to the enzyme if the former proposition is true, and if the latter, it would allow the soluble products to be removed in the washing procedure. There still remains the problem of the failure of the nucleolar shell to respond to the Feulgen reaction. It must be assumed that some other base-combining agent besides a desoxyacid complex is present. It would be interesting to determine the dicarboxylic amino acid content of this structure in the light of the above findings.

The reactions of young pycnotic oocytes to the agents utilized have been described in some detail. Cowdry (1948) says the following regarding pycnosis: "Information is needed on the cause or causes of pycnosis and on the fate of cells in this condition."

It has been noted that the pycnosis cannot fall under the classification of "fatty degeneration," as no osmiophilic bodies are noted in degeneration oocytes. The suggestion was made that the nuclei resembles those of a normal cell in late prophase. Observations on the physiology of the dividing cell suggest the possibility that cellular death could well occur during that period. Quantitative studies on the metabolic rates of dividing cells have shown that the O₂ uptake of the cell is at the
very lowest during late prophase (Stern and Kirk, 1948). The cause for this drop is suggested by the works of Brachet and co-workers (1947 b) who have found that the ribonuclease-removable basophilia drops strikingly in the cytoplasm of cells approaching mitosis. They have further observed that the cytoplasmic ribose nucleic acid is normally associated with discrete particles in the cell which are rich in the respiratory enzymes. These bodies have been found to require the presence of the ribose acid to function normally. Thus the cell, being at a low ebb of metabolic activity, would be particularly sensitive to minor changes occurring in its supply of basic metabolic requirements. Associated with this degeneration is shrinkage of the cytoplasm not evidenced by normal cells and an increase in both cytoplasmic and nuclear basophilia. The entire basophilia of the cytoplasm is removed with ribonuclease and much of the nuclear basophilia not associated with the chromosome-like bodies. This is in line with the observations of Brachet's co-workers (1947 b) that degeneration is accompanied with the transformation of deoxyribose acids to the ribose type. Final stages of degeneration show dense, spherical bodies of the deoxy-nucleic acid complexes. The fate of these bodies has not been determined.

The above description is generally applicable to the
degeneration of follicle cells, except that later stages show intense deposits of osmophilic substances.

SUMMARY

The histophysiological changes in the ovary of prepubertal rats have been determined by cytochemical methods which involve tests for various types of basophilia.

The localization of actively proliferating regions of the germinal epithelium in areas of exogenous concentrations of ribose nucleic acid suggests that complexes of this compound or their degradation products may be instrumental in stimulating mitotic activity in these regions.

The differentiation of the oocyte into a typical protein secreting cell of intense activity appears to be due to an inhibition, the nature of which is undetermined, of the ability of the cell to synthesize desoxyribose nucleic acid.

The nature of the structural composition of the nucleoli has been discussed in line with certain cytochemical findings which suggest that the outer shell is made up of a mild concentration of desoxyribose nucleic acid complex along with some other highly basophilic compound.

It has been suggested that the onset of pycnosis occurs during mitosis, as the cell is metabolically at a
low ebb during that period. Indications of the conversion of the desoxy acid to the ribose type during degeneration are presented.
BIBLIOGRAPHY


EXPLANATION OF FIGURES

All figures are photomicrographs of developing rat ovaries of the ages indicated. All pictures were taken with the aid of a K₂ yellow filter on panchromatic film. Standard 16 mm., 4 mm. and 1.8 mm. objectives with the appropriate Bausch and Lomb Ampliplan oculars were used. Unless otherwise stated staining is methylene blue. Abbreviations used on the figures are as follows:

a. -- antrum
c.t. -- connective tissue
cap. -- capsule
foll.c. -- follicle cell
f.f. -- unilaminar follicle
g.e. -- germinal epithelium
h. -- hilus
met. -- metachromatic bodies
mast. -- mast cell
nc. -- nucleus
ncl. -- nucleolus
ost. -- ostium
od. -- oviduct
per. -- perinuclear band
pyc. -- pyknotic cell
s.l. -- suspensory ligament
s.l.d. -- suspensory ligament, diaphragmatic portion
s.l.p. -- suspensory ligament, proprium portion
th. -- theca

Figure 1. Ovary E17, age 15 d., 9 hr. p.p.
                Vertical section along suspensory ligament. X 40

Figure 2. Ovary E12, age 1 d., 10 hr. p.p.
                Control to figure 3. X 45
Figure 3. Section adjacent to that in figure 2. Processed similarly except for 3 hours ribonuclease treatment. X 45

Figure 4. Ovary E2, age 5 d., 9 hr. p.p. Transverse section through suspensory ligament. X 40

Figure 5. Ovary E21, age 16 d., 10 hr. p.p. Concentration of oocytes and young follicles near ostium. X 50

Figure 6. Ovary E1, age 4 d., 9 hr. p.p. Treated as in figure 2. Control section to figure 7. X 230

Figure 7. Section adjacent to above. Processed as in figure 3. X 230

Figure 8. Ovary E21. Active region of germinal epithelium with underlying young oocytes. X 600

Figure 9. Ovary E21. Similar to above but after ribonuclease treatment. X 600

Figure 10. Ovary E32, age 8 d., p.p. Young follicles with basophilic inclusions in cytoplasm of ovum. Mast cell. X 600

Figure 11. Ovary E21. Follicles and extensive interstitial tissue. X 260

Figure 12. Ovary E21. Multilaminar follicle with oocyte showing intensely staining nucleoli, faint nuclear basophilia and slight cytoplasmic perinuclear basophilic band. X 550

Figure 13. Ovary E21. Feulgen reaction with faintly staining nucleoli and nucleus. X 750

Figure 14. Ovary E21. Ribonuclease effect on nucleoli of adjacent ova. X 240

Figure 15. Ovary E21. Effect of ribonuclease on nucleolus. X 640
Figure 16. Ovary E21. Metachromic inclusions in follicles. X 600

Figure 17. Section adjacent to above. Ribonuclease treated. X 600

Figure 18. Ovary E31. Portion of atretic follicle and egg hill. X 250

Figure 19. Section adjacent to above. Feulgen treated. X 250

Figure 20. Ovary E31. Atretic follicle. Feulgen reaction. X 250

Figure 21. Ovary E12. Fucnosis of young oocytes. X 600

Figure 22. Section adjacent to above. Ribonuclease treated. X 600

Figure 23. Similar to figures 21 and 22. Feulgen reaction. X 600
Figure 12.

Figure 13.

Figure 14.

Figure 15.